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Identification and expression analysis of BTH induced genes in papaya

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Abstract

BTH induces elevated hydrolytic enzyme activity, expression of two members of the PR1 gene family, and resistance to *Phytophthora palmivora* in papaya. Twenty five additional papaya genes showing elevated systemic expression 3 days after BTH treatment have now been identified by suppression subtraction hybridization, and confirmed and quantified by northern blots and quantitative RT-PCR. These genes include several PR genes and related genes known from other systems to have direct anti-microbial activities, and two genes with likely involvement in altering cell wall porosity and lignification. Additionally, six genes with potential roles in establishing reducing conditions following the oxidative burst are induced, including three not previously known to have defense related roles: CPBI 6, a 2-oxoglutarate-dependent oxygenase (2OG-Fe(II) oxygenase), CPBI 14, a malate oxidoreductase, and CPBI 16, a hydroxyphenylpyruvate dioxygenase (HPPDase). Specific Cys residues required for redox activation of arabidopsis NPR1 are conserved in papaya NPR1. Together with the induction of the several genes with potential roles in establishing reducing conditions, this suggests that the regulation of cellular redox status may play a role in SAR induction in papaya.
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Keywords: Benzothiadiazole; *Carica papaya*; PR genes; Quantitative reverse-transcription PCR; Suppression subtractive hybridization; Systemic acquired resistance

1. Introduction

Papaya is an important crop in the Hawaiian economy, and in other tropical areas. Papaya can play an important role in providing human vitamin nutrition and because of its small genome size and relatively short time to maturity, it has been proposed as a model plant system. A high quality BAC library has been produced [32], parts of the genome have been mapped with high density markers [29], and other significant efforts to produce genomic tools are underway (R. Ming, personal communication). Papaya engineered for resistance to papaya ringspot virus [17] was among the first 'GMO' fruit crops to reach the marketplace, and so has been studied extensively, by farmers, consumers, and scientists, as an example of applied biotechnology.

Papaya is subject to attack by a number of bacterial, fungal, and oomycete pathogens [35]. Management and manipulation of endogenous defense responses provide potentially attractive methods of protection against the diseases caused by these pathogens, especially as some existing control methods become illegal and/or less acceptable to consumers. Systemic acquired resistance (SAR) is an inducible defense response found in a large range of plant species including papaya, and like other studied species, papaya SAR can be induced by exogenous application of salicylic acid (SA) or related molecules like benzothiadiazole (BTH). We previously showed that BTH

Abbreviations: BTH, benzothiadiazole; CPBI, *Carica papaya* BTH-induced genes; Cys, cysteine; E, efficiency of amplification; JA, jasmonic acid; PR, pathogenesis-related; qRT-PCR, quantitative reverse-trancription polymerase chain reaction; SA, salicylic acid; SAR, systemic acquired resistance; SSH, suppression subtractive hybridization.

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root treatment induces elevated chitinase and β-glucanase activities in papaya leaves, and also increases resistance to *Phytophthora palmivora* [50]. In this earlier work, we also isolated an NPR1 gene (CpNPR1) isolated by homology to a conserved region of arabidopsis and tobacco NPR1 genes, and four partial pathogenesis related protein 1 (PR1) cDNAs from papaya identified by homology to a region conserved in all known PR1 gene family members. The PR1 genes were designated CpPR1a,b,c and d in the order of discovery as per convention for PR genes in other plant species [44]. CpPR1b and d are induced by BTH, and CpPR1d, which has the highest level of identity to tobacco PR1a, was proposed as a convenient marker of SAR induction [50].

To begin assembling a global picture of SAR in papaya, we now report a set of genes which are systemically induced 3 days after BTH treatment. Characterization of these genes will increase our understanding of papaya defense responses, and can provide a set of tools to facilitate evaluation of disease preventing treatments or varieties.

Suppression subtractive hybridization (SSH) is an approach to global analysis of differentially expressed genes that does not require prior sequence information. The SSH method is designed to produce cDNA libraries enriched for differentially expressed genes and equalized in abundance representation [12]. SSH was used successfully to isolate rice cDNAs induced by jasmonic acid (JA), BTH, or infection with a blast fungus, producing 34 confirmed unique ESTs altered by one or more of the treatments [49]. In tomato, 82 unique cDNAs upregulated in a 35S-*Pto* transgenic line were identified by SSH [48]. In arabidopsis, eight SSH cDNA libraries were produced to identify genes induced by various stress factors, including bacterial and oomycete pathogens, SA and JA. In all 1,058 differentially expressed genes were identified [30]. In barley two genes were isolated which are induced by rust challenge in a non-host barley cultivar, but are not induced in a susceptible cultivar. Additionally a gene specifically induced during successful infection of the susceptible cultivar was isolated [34]. Recently SSH was utilized to obtain 47 unique rice cDNAs whose expression was altered in response to challenge with a blast fungus [28].

While SSH allows global analysis of gene expression without requiring prior sequence data, it does not provide quantitative expression data. In an SSH library a given EST may appear numerous times, however, because SSH normalizes abundance of ESTs in the library, the frequency of a given EST in the library cannot be used to estimate expression levels. Quantitative reverse-transcription PCR (qRT-PCR) can be used to analyze expression of many genes in a given RNA population simultaneously. The sensitivity and accuracy of qRT-PCR expression measurements significantly exceeds that of hybridization based methods. In a comparative study of 1465 arabidopsis transcription factor genes, qRT-PCR was found to be more sensitive, more accurate, and to have greater dynamic range than micro array hybridization data for the same genes [7]. Transcript levels for 83% of the tested genes were measurable by qRT-PCR, while only 55% of the same genes could be measured by micro-array hybridization. The range of transcript levels was estimated to be more than one hundredfold lower in micro-array data than in qRT-PCR data.

When using qRT-PCR, relative quantities of specific nucleic acids are often estimated by the 'comparative $\Delta\Delta C_T$' method [1]. This method assumes the gene of interest has the same amplification efficiency as the endogenous reference gene. If this is not the case, then a dilution series can be used to determine amplification efficiencies or directly as a standard curve. These methods are expensive and time consuming when numerous amplicons are being analyzed. An alternative is to determine amplification efficiencies directly from log fluorescence vs cycle number data [7,37]. The slope of this plot during exponential amplification can be used to calculate amplification efficiency; these calculations can be quickly and easily done with the use of a freely available computer program, LinRegPCR [39].

There are currently very few papaya gene sequences in the public databases, and micro arrays for papaya are not available. For these reasons we chose to test SSH as our approach to identification of differentially expressed genes. Having identified genes putatively induced by BTH treatment, quantitative estimates of changes in expression of these candidate genes were made by northern blots and qRT-PCR. Genes isolated by SSH and confirmed to be differentially expressed in response to BTH were designated *Carica papaya* BTH induced genes (CPBI).

2. Materials and methods

2.1. Plant growth

Carica papaya L. cv. Sun Up seeds were germinated in greenhouse in flats containing potting soil. Three weeks after germination, when seedlings reached approximately 2 cm in height, they were transplanted individually into 4-inch pots containing the same potting soil. Plants were grown in the greenhouse at Aiea, Hawaii, during the winter with temperatures of 20–26 °C and daylight of approximately 12 h. Three-month-old plants were treated and harvested for RNA isolation.

2.2. BTH treatment

BTH (Syngenta) was applied as a suspension of the formulated wettable powder (50% active ingredient). For SSH RNA populations, the suspension was applied as a 500 μM foliar spray (to run-off). This high concentration is likely to have toxic effects, however, none were apparent by the time of harvest (3 days). For reverse northern, northern, and qRT-PCR confirmation and analysis, plants were

treated with 100 μM root drench, which has no detectable effects on long term growth of papaya [50]. Deionized H_2O was used as the negative control treatment.

2.3. RNA isolation

For cDNA synthesis, large scale isolations were performed with 30 g leaf tissue, ground to a fine powder under liquid N_2, and extracted by the acid guanidinium thiocyanate-phenol-chloroform method [5]. Poly-A RNA was isolated with the PolyATtract system (Promega). For northern blots and qRT-PCR, small scale RNA isolations were performed with 100 mg leaf tissue, ground to a fine powder under liquid N_2, and extracted by the method of Bugos et al. [2].

RNA was treated with RQ1 DNase (Promega) per manufacturer's protocol to remove genomic DNA contamination. The dissolved RNA was stored at −80 °C. RNA concentration was estimated after DNase treatment using the RiboGreen RNA Quantitation kit (Molecular Probes) and a Fluorolite1000 (Dynex Technologies) fluorescence plate reader.

2.4. Construction of SSH cDNA library

The PCR-Select subtraction cDNA library kit (CLONTECH) was used to construct the subtracted cDNA library following the manufacturer's protocol, except that the between step purifications were carried out by GENECLEAN SPIN (BIO101) instead of phenol:chloroform extractions. Two μg of mRNA from BTH treated plants (entire aerial part of plant) served as template for tester cDNA synthesis and 2 μg of mRNA from H_2O treatment served as template for driver cDNA synthesis. PCR amplification was conducted using the Advantage cDNA Polymerase Mix (CLONTECH). The final PCR products were cloned into pCR 2.1-TOPO (Invitrogen). After transformation, *E. coli* TOP10F' (Invitrogen) were plated onto Luria-Bertani medium (LB, 10 g tryptone, 5 g yeast extract, 10 g NaCl per l, pH 7.0) containing 100 μg /ml carbenicillin with X-gal and IPTG for blue-white screening.

2.5. Reverse northern dot–blot hybridizations

Nested PCR primer 1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and nested PCR primer 2R (5′-AGCGTGGTCGCGGCCGAGGT-3′) (PCR-Select subtraction cDNA library kit, CLONTECH) were used to amplify the inserts from white colonies in 25 μl PCRs. Three μl of each PCR was denatured at 100 °C for 5 min and applied with a dot–blot manifold onto duplicate Hybond-N^+ (Amersham) membranes. After air-drying, DNA was cross-linked to membranes in a UV Stratalinker 1800 (STRATAGENE). Five hundred nanograms poly-A RNA isolated from leaves 3 days after H_2O and BTH treatments were used to synthesize ^{32}P-labeled first-strand total cDNA probes, which were hybridized to the duplicate sets of membranes at 65 °C as described by Church and Gilbert [6]. After stringency washes, membranes were exposed to X-Omat AR (Kodak) film at −80 °C using Quanta III (DuPont) intensifying screens.

2.6. RNA gel-blot hybridizations

Ten μg total RNA per sample was separated on a 1.5% agarose formaldehyde denaturing gel, then transferred to Hybond N+ membranes (Amersham) by capillary transfer as described previously [42]. Hybridization and stringency washes were performed as described previously [6]. DNA probes were ^{32}P labeled by the random priming method [16]. Washed blots were exposed to storage phosphor screens and read on a GS505 Molecular Imaging System (Bio-Rad). Actin or 18S ribosomal probes were used as loading controls. Integrated pixel densities for BTH (normalized to the loading control) divided by H_2O (normalized to the loading control) hybridization signals were used to estimate induction levels.

2.7. Quantitative RT-PCR

Total RNA was extracted from leaves of five three-month-old seedlings 3 days after BTH or H_2O treatments. Four biological replicates were performed, with the first replicate consisting of five plants in each treatment group, the subsequent three replicates consisting of three plants in each treatment group. Two μg of DNase treated total RNA was used for first strand cDNA synthesis in a 100 μl reaction using the Taqman reverse transcription reagents kit (Applied Biosystems) per manufacturer's protocol. The resulting cDNA was diluted with 450 μl H_2O, and 8 μl per 50 μl reaction or 4 μl per 25 μl reaction of this 5.5-fold dilution was used as template for PCR. Replicate RNA extractions/cDNA syntheses produced highly similar qPCR results; however, all PCRs for a given biological replicate were performed on the same cDNA template pool. PCR was performed with Platinum SYBR Green qPCR Supermix UDG (Invitrogen) with 0.6 μM each primer on a 7900HT Sequence Detection System (Applied Biosystems) or Opticon2 Continuous Fluorescence Detection System (MJ Research). Cycling profile was 50 °C, 2 min; 95 °C, 10 min; 40 cycles of 95 °C, 15 s; 58 °C, 20 s; 72 °C, 30 s; followed by denaturation for melting curve analysis. The fluorescence threshold for determining C_T was set at 0.15 for all experiments. Each PCR was replicated (twice for 50 μl reactions, three times for 25 μl reactions) and normalized to actin. Primers were designed as balanced pairs of 60 °C Tm to amplify fragments of 100–200 bp with Primer Designer 5 (Scientific and Educational Software). Primer sequences for CPBI genes are available upon request. Primers for CpPR1-a,b,c,d and CpNPR1 were as described [50].

2.8. Data analyses

For qRT-PCR analysis, papaya plants were assigned to H_2O or BTH treatment groups in a randomized complete block design. QRT-PCR data for mRNA levels were compared as the means of two (biological replicate 1) or three (biological replicates 2–4) technical (i.e., PCR amplification) replicates. Calculation of gene expression changes from qPCR data was by three methods: first the 'Comparative C_T' method as described [1]. Briefly, for each gene and treatment, ΔC_T was derived by subtracting C_T actin from C_T of each tested gene. $\Delta\Delta C_T$ was derived by subtracting ΔC_T H_2O treatment from ΔC_T BTH treatment for the gene being analyzed. Induction, normalized to actin, was determined by evaluating $2^{-\Delta\Delta C_T}$. General analysis of variance was carried out for each gene to determine significance of differences in ΔC_T values between treatments and biological replicates [41]. A second method was to determine amplification efficiencies by performing qPCR on serial dilutions of cDNA template. Seven ESTs (actin, CPBI4, 6, 10, 14, 25 and CpPR1b) were chosen for validation experiments with five 2-fold serial dilutions. A plot of C_T vs $\log_2$ of the relative concentrations of starting cDNA could be fit to straight lines ($R^2 \geq 0.90$) for all but one (CpPR1b) of the tested ESTs, yielding five ESTs that could be validated relative to the actin control. This slope was then used to calculate efficiency (E) of amplification by solving $2^{(-1/\text{slope})}$. This E value was then used to calculate a corrected induction ratio with the formula $(E_X^{CT\ H_2O\ X}/E_{actin}^{CT\ H_2O\ actin})/(E_X^{CT\ BTH\ X}/E_{actin}^{CT\ BTH\ actin})$ where X is the gene (EST) being analyzed [38]. The corrected induction values of four of the five genes were reduced relative to the non-corrected induction values; for one gene the corrected ratio is greater than non-corrected. The third method was to estimate amplification efficiencies from the slope of the log fluorescence vs cycle number during the exponential phase where $E = 10^{\text{slope}}$. Slope values were calculated with LinRegPCR [39] freeware described by Ramakers et al. [37]. Slopes were determined with four to six points producing the best correlation coefficients. Slope values with $R^2 \geq 0.999$ were averaged from 18 replicates (biological replicates 2–4, two treatments, three PCRs each) to estimate an average E value for each amplicon. Change in gene expression was calculated as $(E_X^{CT\ H_2O\ X}/E_{actin}^{CT\ H_2O\ actin})/(E_X^{CT\ BTH\ X}/E_{actin}^{CT\ BTH\ actin})$ where X is the gene (EST) being analyzed as described above.

3. Results

3.1. SSH library

To maximize differences between tester and driver mRNA populations, plants for SSH experiments were treated with a high concentration (500 μM) of BTH as a foliar spray. This concentration is likely to have toxic effects on the plants [50], although none was observed at the time of harvest. Leaf RNA was used for cDNA synthesis and SSH. An aliquot of SSH-selected cDNA was ligated to a plasmid vector and used to transform *E. coli*. Three hundred sixty white colonies were obtained on X-gal media. In addition to SAR-related genes this library was likely to include genes induced by BTH toxicity, and genes induced locally but not systemically. Twenty-eight colonies were chosen at random for sequence and northern analysis to assess the quality of the library. Four of these colonies were found to be duplicate clones. Of the 24 unique clones, northern blots indicated 12 were induced by BTH, six were unchanged, and six produced no signals detectable by northern blot with either H_2O or BTH treatments. This small sample indicated the library contained a useful number of BTH induced genes, so the remaining 332 clones were analyzed.

3.2. Reverse northern dot blots

RNA for reverse northern, northern, and qRT-PCR analysis was extracted from leaves of plants treated by 100 μM BTH root drench. This treatment causes minimal visible effects, has no long term effect on growth, and was used in previous experiments [50]. mRNAs elevated in this population are unlikely to represent non-specific BTH effects, and should definitely represent genes that are induced systemically. Replica dot blots for the 332 remaining clones were hybridized first to labeled total cDNA from H_2O and BTH treated seedlings, respectively (Fig. 1). This was followed by hybridization with a combined probe for the originally sampled 28 clones to eliminate duplicate clones. This procedure identified 89 BTH induced clones, which did not cross-hybridize with any of the originally sampled 28 clones. Analysis of these clones identified 15 unique clones, representing 12 or 13 unique genes. In total (including the 12 differentially expressed clones identified in the first sample of 28), 24 or

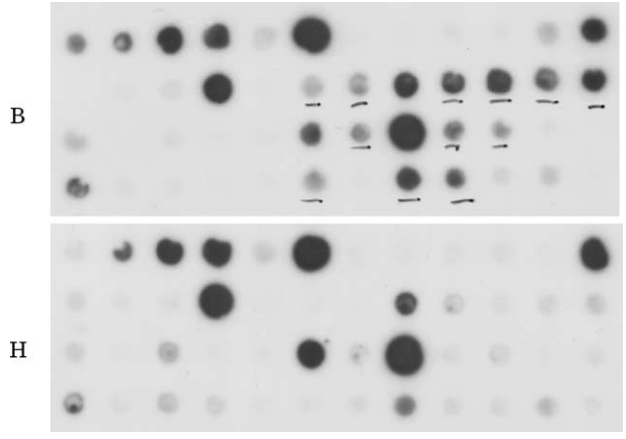

Fig. 1. Representative duplicate reverse northern dot blots of gene induction 3 days following BTH treatment. Dot blots of putative CPBI clones hybridized to labeled total cDNA from plants treated with BTH (B) and H_2O (H) 3 days before harvest.

Table 1
Putative identifications of unique CPBI genes

ID	Access. no	Similarity	Process
CPBI 1	CO373888	Arabidopsis ripening regulated protein	Unknown
CPBI 2	CO373889	Citrus blight-associated protein p12 precursor	Unknown
CPBI 3	CO373890	Grape class IV endochitinase, PR4	Fung cell wall hydrolase
CPBI 4	CO373891	Tobacco peroxidase AB027753.1	Oxidat stress response
CPBI 5	CO373892	Arabidopsis cytochrome P450, putative	Electron transport
CPBI 6	CO373893	Arabidopsis 2OG-Fe(II) oxygenase family	GA biosynth
CPBI 7	CO373894	Soybean ribosome-associated protein p40	Protein biosynth
CPBI 9	CO373895	Arabidopsis RPT2, sig. trans. phototropic resp.	Phototropism
CPBI 10	CO373896	Arabidopsis putative protein	Unknown
CPBI 11	CO373897	*Nepenthes alata* aspartic proteinase 3	Proteolysis
CPBI 12	CO373898	Tomato heat shock cognate protein 80	Protein folding
CPBI 13	CO373899	Grape chitinase III AB105374.1	Fung cell wall hydrolase
CPBI 14	CO373900	*Ricinus communis* NADP-dependent malic protein	Malate metabolism
CPBI 15	CO373901	*N. rustica* PIPK1	Unknown
CPBI 16	CO373902	Arabidopsis 4-hydroxyphenylpyruvate dioxy	Carotenoid biosynthesis
CPBI 17	CO373903	Tomato osmotin precursor AAC64171	Defense
CPBI 18	CO373904	Tomato polyubiquitin	Protein degradation
CPBI 19	CO373905	Arabidopsis pectinesterase-like protein	Cell wall modification
CPBI 20	CO373906	Arabidopsis callose synthase 1 cat. subunit	1,3-β-glucan synthase
CPBI 21	CO373907	Cotton bacterial-induced guaiacol peroxidase	Oxidat stress response
CPBI 22	CO373908	Arabidopsis putative membrane protein	Unknown
CPBI 24	CO373909	Arabidopsis 60S ribosomal protein L11	Protein biosynthesis
CPBI 25	CO373910	Tobacco peroxidase	Oxidat stress response
CPBI 26	CO373911	Rice unknown protein	Unknown
CPBI 27	CO373912	Rice similar to GDSL-motif lipase/hydrolase	Unknown
NPR1	CF588412	Tobacco NPR1	PR gene regulation
PR1a	CF569398	Arabidopsis pathogenesis-related protein, putative	Unknown defense
PR1b	CF569399	Arabidopsis pathogenesis-related protein, putative	Unknown defense
PR1c	CF569400	Arabidopsis pathogenesis-related protein, putative	Unknown defense
PR1d	CF569397	Arabidopsis pathogenesis-related protein, putative	Unknown defense

Process is deduced from AraCyc: *Arabidopsis thaliana* Biochemical Pathways (http://www.arabidopsis.org/tools/aracyc/). Missing numbers (i.e. CPBI 8 and 23) are clones determined to represent new fragments of previously listed genes (i.e. CPBI 3 and 6).

25 (CPBI 4 and 25 may be different fragments of the same gene) unique BTH induced genes were identified. For simplicity, we treat CPBI 4 and 25 as different genes throughout the paper.

3.3. Sequence analysis

Sequence of differentially hybridizing clones revealed 27 unique ESTs, 25 are presented here (Table 1). Three pairs of ESTs were determined to be non-overlapping fragments of similar or potentially the same gene. Subsequent RT-PCR experiments (data not shown) determined that two of these pairs (CpBI 3 and CpBI 8; CpBI 6 and CpBI 23) were indeed fragments from the same transcript. The third pair (CpBI 4 and CpBI 25) was not recovered as a single RT-PCR product, so these may be related but distinct genes. BTH induction of these two ESTs was very similar. Similarity matches in the non-redundant GenBank database were used to assign putative functions.

3.4. Northern blot analysis

Northern blot hybridizations (Fig. 2) confirmed induction for all SSH ESTs determined to be differentially expressed by the reverse northern dot blots. Phosphor imager pixel densities for hybridization bands were integrated and used to calculate fold induction, shown in Table 2. In some cases, visual representations of hybridization signals did not appear to agree with phosphor imager counts, however counts generally produced similar induction values to qRT-PCR estimates for the same gene. In addition to genes identified by SSH, Table 2 contains five genes previously

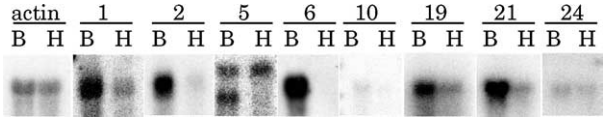

Fig. 2. Representative RNA gel blots of gene induction 3 days following BTH treatment. RNA extracted from leaves following BTH (B) or H_2O (H), root drench, hybridized to probes for actin, CPBI 1 (1), 2, 5, 6, 10, 19, 21 and 24.

Table 2
Expression change estimated by four methods

Gene Actin	Induction				P trtmnt
	Northern	cmp C_T	Dilution	Slope	
CPBI 1	4.53	2.19	nt	1.90	0.05
CPBI 2	12.20	5.83	nt	3.91	0.00
CPBI 3	16.70	11.32	nt	7.53	0.00
CPBI 4	14.60	7.67	5.15	5.98	0.01
CPBI 5	m bnds	0.94	nt	0.96	0.55
CPBI 6	$H_2O=0$	857.48	nt	131.67	0.00
CPBI 7	1.54	1.39	nt	1.29	0.19
CPBI 9	1.80	2.56	nt	2.23	0.00
CPBI 10	1.54	1.58	1.42	1.43	0.04
CPBI 11	1.94	1.68	nt	1.57	0.14
CPBI 12	1.65	1.24	nt	1.19	0.41
CPBI 13	8.23	7.06	nt	4.82	0.00
CPBI 14	8.90	11.83	2.97	3.14	0.02
CPBI 15	3.38	0.51	0.55	0.58	0.02
CPBI 16	4.34	6.88	nt	4.77	0.01
CPBI 17	6.66	68.07	nt	31.55	0.00
CPBI 18	1.54	1.48	nt	1.38	0.10
CPBI 19	1.52	1.41	nt	1.26	0.21
CPBI 20	6.10	2.47	nt	1.97	0.01
CPBI 21	3.73	6.62	nt	4.39	0.01
CPBI 22	1.99	1.20	nt	1.11	0.55
CPBI 24	1.58	1.70	nt	1.52	0.03
CPBI 25	1.51	6.25	5.16	4.64	0.00
CPBI 27	nd	3.26	nt	2.11	0.03
NPR1	nt	1.19	nt	1.06	0.49
PR1a	nt	0.20	nt	0.25	0.00
PR1b	nt	5.89	nt	3.15	0.01
PR1c	nt	0.85	nt	0.82	0.66
PR1d	17.00	16.11	nt	6.99	0.00

P trtmnt, probability of no significant change in gene expression due to BTH treatment; comp C_T, expression change $=2^{-\Delta\Delta C_T}$; dilution, expression change $=(E_X^{CT\ H_2O\ X}/E_{actin}^{CT\ H_2O\ actin})/(E_X^{CT\ BTH\ X}/E_{actin}^{CT\ BTH\ actin})$, where E is determined from a dilution series; slope, expression change $=(E_X^{CT\ H_2O\ X}/E_{actin}^{CT\ H_2O\ actin})/(E_X^{CT\ BTH\ X}/E_{actin}^{CTBTH\ actin})$, where E is determined from log slope fluorescence versus C_T (see Section 2 for details); m bnds, multiple hybridizing bands; nt, not tested; nd, not detected.

isolated by homology to genes known from other systems to be involved in plant defense (NPR1, CpPR1a, CpPR1b, CpPR1c and CpPR1d). Northern hybridization values were normalized to signals for actin. Results for genes induced a minimum of 1.5-fold were reported. Time course experiments with two chitinase genes (CPBI 3 and 13) and a thaumatin-like gene (CPBI 17) showed that mRNA accumulation for these genes increased for 4 days after BTH treatment, and then decreased gradually, while a peroxidase gene (CPBI 4) peaked at 1 day and declined rapidly. In contrast, CpPR1d, previously identified as a SAR marker in papaya [50], continued to increase through 14 days after treatment (Fig. 3). This prolonged period of elevated expression for several genes is more like tobacco [19] than arabidopsis, where numerous BTH induced genes return to basal levels approximately five days after treatment [26]. H_2O controls were carried out for 3 day experiments for all genes, and showed clear differences from BTH treatments (Fig. 2). Because the plants were already 3 months old at the start of the time course

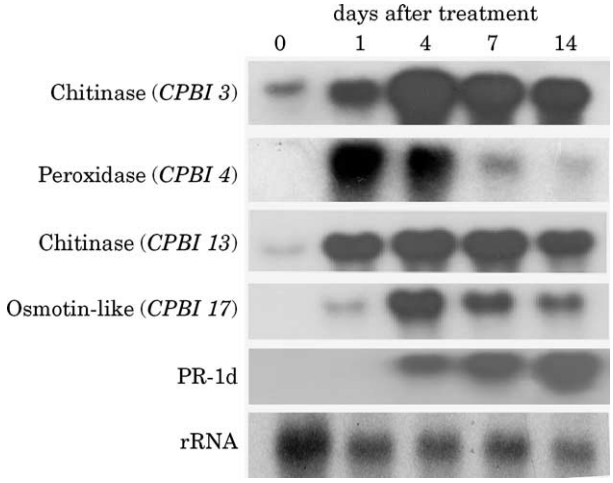

Fig. 3. RNA gel blots of gene induction time course following BTH treatment. RNA extracted from leaves BTH root drench. Previously characterized CpPR1-d for comparison, rRNA for loading control.

experiments, we feel it unlikely the gene expression changes observed over the 14 day time course represent developmental changes unrelated to the BTH treatment, however, we cannot exclude this possibility.

3.5. qRT-PCR

Three parallel extractions from the same plant pool, DNase treatments, and cDNA syntheses produced highly similar qPCR results (a maximum SD of 0.64 cycle for the ΔC_T for CPBI 6 across three parallel experiments, with five dilutions for each experiment). In general, qRT-PCR confirmed BTH induction for most of the tested CPBI genes, although levels of induction were in several cases found to be quite different from that indicated by northern analysis (Table 2). Transcripts for all CPBI genes and five previously cloned defense related genes were detected in both treatment groups. In addition, six genes for which transcripts had not been detected by northern blot were all detectable by RT-PCR, and one of these was induced by BTH (Table 2). Melting curve analysis indicated that each primer pair amplified a single major species, and the T_M of that product was reproduced in all technical and biological replicates. PCR replicate C_T values were highly similar, with 34.5% having SD $\leq$ 0.1 cycle and 98.4% $\leq$ 0.9 cycle. C_T values for the endogenous control gene, actin, were highly similar (24.10 ± 0.51) in all experiments and treatments. Expression change following BTH treatment was calculated by three methods: the comparative $\Delta\Delta C_T$ method, which assumes 100% amplification efficiency, and two methods which incorporate experimentally determined amplification efficiency values (see Section 2). Expression change values as determined by these methods are shown in Table 2.

C_T values were quite consistent across biological replicates. Fig. 4 plots C_T values from BTH treated plants in replicate 1 vs replicate 2. A straight line can be fit to these points with an R^2 value of 0.9625 and with a slope of 1.0091,

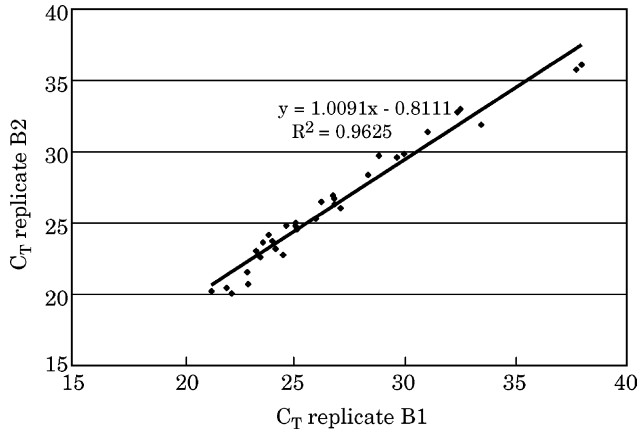

Fig. 4. Reproducibility of CPBI C_T values between biological replicates. C_T values from BTH treatment B1 (X axis) versus BTH treatment B2 (Y axis) for 31 tested amplicons. Each value is the average of three PCRs.

indicating that the biological response and our measurement of the response is highly reproducible.

4. Discussion

Genes induced by avirulent pathogen elicitation or treatment with SA (or INA or BTH) can be grouped into an 'immediate early' set, and those including the PR genes that are induced later. In treated tobacco leaves, some genes are detectably induced within 30 min, while PR1a does not increase detectably until 5–6 h [21]. When tobacco was inoculated with Tobacco Mosaic Virus (TMV), 12 of 13 tested PR and related defense genes were detectably induced within 3 h in inoculated leaves, while systemic leaves were not induced until 6 h and generally peaked at lower levels [45]. In systemic leaves of BTH treated papaya, mRNA for a peroxidase gene has peaked by 1 day, and 2 chitinase genes and an osmotin-like gene have peaked by 4 days. CpPR1d, in contrast, continues to increase through 14 days (Fig. 3). To confirm genes recovered in the SSH library as being BTH induced, mRNA from systemic leaves was sampled at 3 days after treatment. This time point is likely to find elevated mRNA levels for PR type 'later' genes, but may not find immediate early genes that have already returned to baseline levels [21].

Current reviews [13,14,25,47] on the induction of SAR in plants support a model with an oxidative burst and a rapid increase in reactive oxygen species (ROS) occurring as an early event near the site of elicitation. The elevated ROS may have direct antimicrobial effects, and roles in blocking systemic pathogen movement by cross-linking cell wall proteins and promoting lignification. H_2O_2 and perhaps other ROS induce benzoic acid 2-hydroxylase (BA2H) activity, which catalyzes the synthesis of SA from benzoic acid (BA) [27]. Although exogenous application of SA or related molecules can trigger accumulation of ROS and SAR, elicitation by avirulent pathogens triggers elevated ROS before SA becomes elevated [8]. Elevated SA stimulates the lipase activity of an SA-binding protein (SABP2) in tobacco [24]. The initial increase in oxidative state is followed by establishment of a reducing state, which is essential for movement of NPR1 protein into the nucleus [33] and the modification of some TGA transcription factors to induce binding to NPR1 [11]. The reducing state may be induced by SA-mediated, NPR1-independent induction of antioxidant encoding genes including glutathione S-transferase and glucosyltransferase [43].

The BTH-induced genes identified in papaya included several PR genes and related genes known from other systems to have PR-like roles (Table 1).These include two different chitinase genes (CPBI 3 and 13) and an osmotin (CPBI 17), that likely have direct anti-microbial activities. A pectinesterase (CPBI 19) and a guaiacol peroxidase (CPBI 21) have likely involvement in cell wall cross-linking and lignification. In addition to CPBI 21, there are two other

peroxidases (CPBI 4 and 25) and a cytochrome P450 (CPBI 5) that have likely roles in detoxifying ROS and establishing reducing conditions in the cell. Several other genes, to our knowledge not previously known to have defense related roles, also encode proteins with potential roles in detoxifying ROS and establishing reducing conditions in the cell, including CPBI 6, a 2-oxoglutarate-dependent oxygenase (2OG-Fe(II) oxygenase), CPBI 14, a malate oxidoreductase, and CPBI 16, a hydroxyphenylpyruvate dioxygenase (HPPDase). In arabidopsis approx. 100 members of the 2OG-Fe(II) oxygenase gene family have been identified or predicted including members involved in biosynthesis of flavonoids and other secondary metabolites [46]. As of June 2004, there were no micro array datasets in The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/index.jsp) database which showed a two-fold or greater change for any of these genes in plant defense related experiments, however CPBI 6 is induced very significantly by BTH (Table 1). In *A. thaliana* ecotype Shahdara, HPPDase mRNA levels are not significantly altered in leaves inoculated with Tobacco Mosaic Virus at 4 days post inoculation (dpi) or in systemic leaves at 14 dpi [20], however, both PR1 and PR-5 are induced at 3 dpi in this ecotype [9]. In contrast, papaya HPPDase is induced about fivefold 3 days after treatment (Table 1) with BTH, which also induces CpPR1b and d and a PR5, (CPBI 17, an osmotin,Table 1). HPPDase catalyzes the formation of plastoquinone and tocopherols, which have essential roles in scavenging free radicals and thereby shift cellular equilibrium towards a reducing state [36]. The proteins encoded by these three genes potentially all affect the cellular redox state and this has a very important role in the induction of defense response genes. In non-induced arabidopsis, NPR1 protein exists as an oligomer linked by intermolecular disulfide bonds. Establishment of reducing conditions converts NPR1 protein to a monomeric form, which results in NPR1 entering the nucleus and transcriptional activation of PR genes. Mutation of Cys residues 82 or 216 results in constitutive monomerization of the protein [33]. These Cys residues are conserved in the papaya CpNPR1 protein, which suggests that establishment of reducing conditions may also play an important role in papaya SAR induction.

Several of the CPBI genes have potential roles in signal transduction. CPBI 9 has significant similarity to arabidopsis RPT2, which is involved in phototropism signal transduction. The region of high similarity includes a predicted coiled-coil domain at residues 500–530 of RPT2, predicted to mediate protein–protein interactions [40]. CPBI 15 has significant similarity to 1-phosphatidylinositol-4-phosphate 5-kinase (PIP5K) encoding genes. PIP5K phosphorylates phosphatidylinositol 4-phosphate to produce PIP2, the precursor of Inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), both of which trigger calcium fluxes into the cytosol, which occurs as an early event in plant defense signal transduction [8]. PIP5K genes have been shown to be induced by abiotic stress in plants, including mung bean [23] and arabidopsis [31]. CPBI 27 is predicted to encode a GDSL-motif lipase. A lipase of this type is induced in tomato by the fungal toxin fusicoccin [18]. Arabidopsis EDS1 [15] and PAD4 [22] encode different types of lipase enzymes, but both are involved in pathogen defense signaling. Recently, SA-binding protein 2 (SABP2) of tobacco was shown encode a lipase. SABP2 binds SA with considerably greater affinity than other candidate SA 'receptors', and lipase activity of the protein is increased by SA. Silencing of SABP2 inhibits both local and systemic disease resistance responses; authors postulate that SABP2 may be a key 'resistance-signaling receptor for SA' and may produce a lipid derived signal molecule [24]. SABP2 does not have obvious sequence similarity to EDS1, PAD4, or CPBI 27.

In addition to CPBI genes with proposed functions in disease resistance, other genes were obtained without known functions. CPBI 2 has high similarity to citrus P12. Citrus P12 accumulates in trees with citrus blight, an important disease of unknown etiology. The function of P12 is unknown, although it shares considerable identity with expansin proteins [4,10]. CPBI 1, 10, 22, and 26, have similarity only to genes or predicted genes of unknown function.

While both expected and unexpected differentially expressed genes were isolated from papaya by SSH, some genes induced by BTH were not recovered in this SSH sample. These include CpPR1b and CpPR1d both of which are significantly induced by BTH. Construction of an SSH library with the PCR-Select protocol is dependent on the presence of at least one *Rsa*I recognition site in the cDNA; while this four base sequence is likely to be present in most cDNAs, those lacking *Rsa*I sites will not be recovered in the library. The partial cDNA sequences we have for CpPR1b and CpPR1d do not contain the *Rsa*I recognition sequence; if the sequence of the remainder of these genes also does not contain *Rsa*I, this would explain their absence in the library. CpNPR1 does contain three *Rsa*I sites and our initial analysis [50] indicated the gene was induced by BTH, albeit only 1.7-fold. This is consistent with the low level of induction (approximately 2-fold) observed in arabidopsis [3]. The three additional biological replicates reported in this paper indicate the low level of induction observed in papaya is not statistically significant. Furthermore C_T values for CpNPR1 in BTH treated plants are higher than for any of the tested genes recovered in our SSH library, indicating the CpNPR1 mRNA is a relatively rare message even after BTH treatment. This low abundance and/or low level induction may explain the absence of CpNPR1 in our library. In the end SSH is a sampling method, and the absence of these genes underscore that a single iteration of the process does not yield complete results. Repeating SSH, or analyzing another aliquot of the SSH PCR products could yield additional genes. The high percentage of the library that hybridized to the first set of 24 unique clones, however, indicates that additional clones would include an

ever-increasing percentage of duplicates. A high degree of redundancy in SSH libraries has also been observed by other researchers [28,49]. Mahalingham and co-workers [30] plotted number of SSH clones sequenced versus number of genes identified and concluded that "…by the time several hundred clones had been examined, few new genes remain to be identified in a given library."

Our data suggest a possible role for the establishment of cellular reducing conditions in papaya SAR induction, and that the nuclear mobilization of CpNPR1 may be controlled by redox state, as has been demonstrated for arabidopsis [33]. Genes with potential redox roles in papaya include several shown to be defense related in other systems, but also three previously not reported to have this role. Other papaya genes with novel roles in plant defense include potential signal transduction effectors.

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